Depletion of CD4$^+$ Cells Exacerbates the Cutaneous Response to Acute and Chronic UVB Exposure

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Solid organ transplant recipients have a 60–250-fold increased likelihood of developing sunlight-induced squamous cell carcinoma (SCC) compared with the general population. This increased risk is linked to the immunosuppressive drugs taken by these patients to modulate T cell function, thus preventing organ rejection. To determine the importance of T cells in the development of cutaneous SCC, we examined the effects of selectively depleting Skh-1 mice of systemic CD4$^+$ or CD8$^+$ T cells, using monoclonal antibodies, on ultraviolet B (UVB) radiation-induced inflammation and tumor development. Decreases in systemic CD4$^+$ but not CD8$^+$ T cells significantly increased and prolonged the acute UVB-induced cutaneous inflammatory response, as measured by neutrophil influx, myeloperoxidase activity, and prostaglandin E_2 levels. Significantly more p53$^+$ keratinocytes were observed in UVB-exposed CD4-depleted than in CD4-replete mice, and this difference was abrogated in mice depleted of neutrophils before UVB exposure. Increased acute inflammation was associated with significantly increased tumor numbers in CD4-depleted mice chronically exposed to UVB. Furthermore, topical treatment with the anti-inflammatory drug celecoxib significantly decreased tumor numbers in both CD4-replete and CD4-depleted mice. Our findings suggest that CD4$^+$ T cells play an important role in modulating both the acute inflammatory and the chronic carcinogenic response of the skin to UVB.

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INTRODUCTION

Although less than 1–2% of the ultraviolet (UV) light from the sun is in the 290–320 nm spectral range, UVB radiation is responsible for the majority of cutaneous damage following both acute and long-term exposure (Brash *et al.*, 1991; Jung, 1991). Exposure to UV radiation induces functional changes in both resident skin cells as well as infiltrating cells of the immune system, which ultimately contribute to the development of skin cancer (Elmets and Bergstresser, 1982; Kripke, 1984, 1991; Yamawaki *et al.*, 1997). Exposure to UVB light is initially associated with an inflammatory response characterized by increased blood flow and vascular permeability, which results in edema and erythema, the infiltration of neutrophils into the dermis and the induction of pro-inflammatory cytokines and prostaglandins (Black *et al.*, 1978; Gilchrest *et al.*, 1981; Jung, 1991; Rivas and Ullrich, 1994). It is now clear that inflammatory cells have significant effects on tumor development. Early in the tumor development process, inflammatory cells such as neutrophils can be powerful tumor promoters. Neutrophils and other phagocytic cells induce DNA damage in proliferating cells through the generation of antimicrobial reactive oxygen and nitrogen species (Dedon and Tannenbaum, 2004). In addition, these inflammatory cells generate arachidonic acid derivatives, including prostaglandins and leukotrienes, which exacerbate the inflammatory response (Malech and Gallin, 1987). We have demonstrated previously that modulating UVB-induced prostaglandin E_2 (PGE_2) production via the topical application of the COX-2-inhibitor celecoxib, inhibited both UVB-induced acute inflammation, including neutrophil infiltration and UVB-induced tumor development (Wilgus *et al.*, 2000, 2003). These studies, taken together with studies carried by others, support a link between inflammation and tumor development in the skin and other organs (Coussens and Werb, 2002; Clevers, 2004).

Organ and therefore patient survival following transplantation have increased substantially over the past 20 years as a result of better surgical techniques and more effective immunosuppressive regimes. Concomitant with this increased survival rate, there has been an increased incidence of various cancers in these patients. Non-melanoma skin cancer (NMSC) is presently the most common post-transplant malignancy (Bavinck *et al.*, 1993; Webb *et al.*, 1997). Compared with the general population, solid organ transplant recipients have a 60–250-fold increased likelihood of developing UVB-induced squamous cell carcinomas (SCC) that are likely to metastasize (Hartevelt *et al.*, 1990; Jensen *et al.*, 1999; Lindelof *et al.*, 2000). In fact, SCC has been

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reported to account for 27% of deaths in Australian heart transplant patients surviving more than 4 years after transplantation (Ong *et al.*, 1999). The presence of signature mutations in the p53 gene characteristic for UV light in skin tumors arising in immunocompromised patients indicates that, as in the general population, UV light is also an important etiologic factor for the development of these tumors (McGregor *et al.*, 1997).

The increase in skin tumor risk seen in transplant recipients is related to the systemic immunosuppressants these patients must take to prevent rejection of the new organ, as lower immunosuppressant levels are correlated with lower skin cancer incidence (Otley *et al.*, 2001). To a lesser extent, an increase in SCC risk also occurs in other immunosuppressed individuals, including patients with chronic lymphocytic leukemia (CLL), non-Hodgkin's lymphoma, and HIV (Levi *et al.*, 1996; Nguyen *et al.*, 2002; Hemminki *et al.*, 2003). Multiple clinical phenomena highlight the close relationship between cutaneous SCC risk and immunity. For example, actinic keratoses, benign lesions that can progress to SCC, are known to regress spontaneously in some immunocompetent patients with prolonged photoprotection, presumably via immune mechanisms (Frost and Green, 1994).

A common feature of the diseases displaying significant increases in SCC development is suppression or dysregulation of $CD4^+$ T cell function. It has been reported that 23% of transplant patients have reduced circulating levels of $CD4^+$ T cells (Hutchinson *et al.*, 2003) and Ducloux *et al.* (1998) reported that transplant recipients with skin cancer had significantly lower $CD4^+$ T cell counts compared with transplant recipients without skin cancer. CLL patients at increased risk for SCC have a low CD4/CD8 T-cell ratio and severely dysregulated T-cell function (reviewed in Mellstedt and Choudhury, 2006). A report describing recurrence of SCC in a CLL patient on treatment with a T-cell-depressant further supports an important connection between T cell function and SCC risk (Davidovitz *et al.*, 1997). Furthermore, anecdotal evidence shows an association with CD4 leukopenia and Bowen's disease (SCC *in situ*) (Hayashi *et al.*, 1997). Similarly, the progressive depletion and dysfunction of $CD4^+$ T cells associated with HIV infection seem likely to explain increased risk of SCC in HIV/AIDS patients (Wilkins *et al.*, 2006). Thus, various primary immune defects in T cell function occurring in disease states or following immunosuppressive therapy may result in increased SCC development.

In this study, we used the Skh-1 hairless mouse model of UVB-induced inflammation and carcinogenesis to examine the effects of $CD4^+$ T cell depletion on the cutaneous response to both acute and chronic UVB exposure. We used immunohistochemical analysis and a biochemical assay to determine changes in the levels of UVB-induced neutrophil infiltration into the skin as one marker of changes in inflammation. The levels of PGE_2, a potent mediator of the UVB-induced inflammatory response in the skin and a key mediator in the development of UVB-induced skin tumors, were also evaluated (Wilgus *et al.*, 2000, 2003). P53 stabilization as indicated by nuclear immunoreactivity was used as an indicator of DNA damage (Selivanova, 2002). We report that $CD4^+$ T cells infiltrated into the skin following UVB exposure and that the specific depletion of $CD4^+$ T cells increased both the acute UVB-induced inflammatory response, as well as the number of UVB-induced tumors. Furthermore, topical treatment with the anti-inflammatory drug celecoxib significantly decreased tumor numbers in $CD4^+$ T-cell depleted mice, supporting the link between the enhanced inflammation seen in the immunocompromised animals and tumor development. This study suggests that the $CD4^+$ lymphocyte, a cell type clearly affected by both disease-related and therapeutic immunosuppression, has important roles in modulating UV-induced inflammation and protecting against UV-induced skin tumor development.

RESULTS

Cutaneous T cell infiltration following acute UVB exposure is altered by specific T cell depletion

To examine the effect of systemic CD4 or CD8 depletion on $CD4^+$ and $CD8^+$ T cell infiltration into the skin in response to acute UVB exposure, Skh-1 hairless mice ($n=10$ per group) were injected intraperitoneally (i.p.) with anti-CD4, anti-CD8, or IgG control antibodies. Mice were exposed to UVB on non-consecutive days for a total of three exposures; skin was harvested 24 hours after the last exposure. The numbers of $CD4^+$ or $CD8^+$ cells were quantitated by immunohistochemistry. As demonstrated in Figure 1a, exposure to UVB resulted in a statistically significant ($P<0.02$) increase in the number of $CD4^+$ T cells in the epidermis of mice injected with the control IgG antibody compared with non-UVB-exposed IgG-injected mice. Selective depletion of CD4 cells with anti-CD4 antibodies caused a statistically significant ($P<0.04$) decrease in the number of $CD4^+$ T cells infiltrating into the epidermis following UV exposure. Selective depletion of $CD8^+$ cells had no effect on UV-induced $CD4^+$ T cell infiltration of the skin.

As expected, selective depletion of $CD8^+$ cells with anti-CD8 antibodies resulted in a statistically significant ($P<0.001$) decrease in the number of $CD8^+$ T cells infiltrating into the epidermis (Figure 1b). UVB exposure alone did not increase $CD8^+$ cell numbers in the epidermis. However, selective depletion of $CD4^+$ cells resulted in a significant increase in $CD8^+$ cells ($P<0.0006$) in the epidermis.

UVB-induced myeloperoxidase levels are increased in skin from CD4-depleted mice

Increased neutrophil number and activity are a hallmark of the acute UVB-induced inflammatory response in the skin. Myeloperoxidase (MPO) is an enzyme produced by neutrophils that converts hydrogen peroxide to hypochlorous acid. MPO activity reflects the level of neutrophil activation in tissues and is an accurate measure of cutaneous inflammation. As we have reported previously (Wilgus *et al.*, 2000), exposure to UVB (UV/IgG) resulted in a significant increase ($P<0.01$) in cutaneous MPO levels as well as neutrophil numbers compared with the

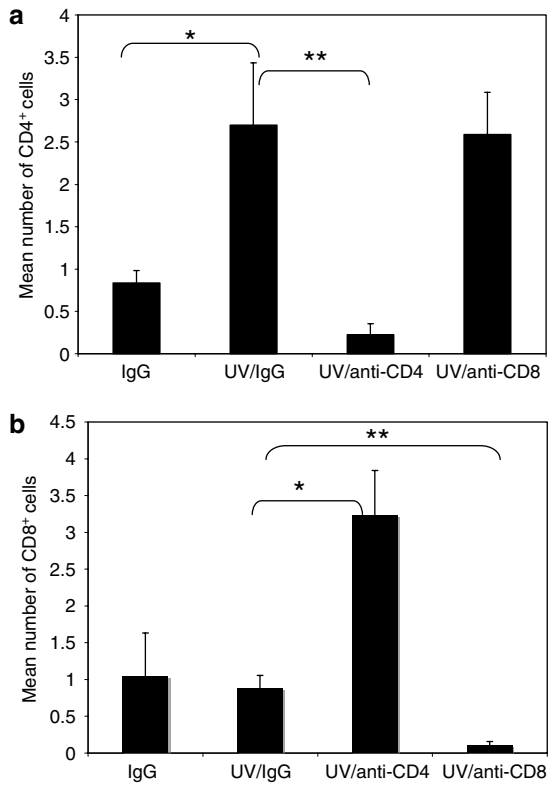

Figure 1. UVB exposure increases epidermal infiltration of $CD4^+$ but not $CD8^+$ T cells. Skh-1 hairless mice were selectively depleted of $CD4^+$ or $CD8^+$ cells before UVB as described in the Materials and Methods section. Quantitation of immunohistochemically stained sections ($n=10$ per group) was carried out to determine the number of epidermal infiltrating $CD4^+$ (**a**) or $CD8^+$ (**b**) cells. Error bars indicate SEM. **(a)** $*P<0.02$; $**P<0.04$; **(b)** $*P<0.0006$; $**P<0.001$.

non-UVB-exposed control skin (IgG; Figure 2a–d). A significant increase in MPO ($P<0.004$) as well as neutrophil number ($P<0.04$) was observed in the skin of UVB-exposed CD4-depleted animals (UV/anti-CD4) compared with control IgG-injected UVB-exposed animals (Figure 2a–d). There was no significant difference between MPO levels or neutrophil numbers in the skin of CD8-depleted (UVV/anti-CD8) versus control IgG-treated animals exposed to UVB.

We also examined the effect of CD4 depletion on MPO levels at 24 hours, 48 hours, and 1 week following a single exposure to UVB. As shown in Figure 2b, peak levels of MPO were achieved in skin isolated from IgG-injected mice at 48 hours following a single UVB exposure. Selective depletion of $CD4^+$ cells not only increased MPO levels at both 24 and 48 hours compared with controls ($P<0.001$ and $P<0.004$, respectively), but also prolonged the observed increase in MPO levels through 1 week ($P<0.005$).

CD4 depletion increases cutaneous PGE_2 levels and enhances p53 induction within the epidermis in UVR-exposed skin

The effects of UVB exposure and CD4 depletion on PGE_2 levels were examined following 1 week of UVB exposure. Mice received three UVB exposures on non-consecutive days and skin was harvested 24 hours after the last exposure. Levels of PGE_2 in UVB-exposed control IgG-injected mice (UV/IgG) were significantly increased compared with levels in unirradiated controls (IgG; $P<0.002$) (Figure 3a). Depletion of $CD4^+$ cells significantly enhanced UVB-induced PGE_2 production ($P<0.006$).

Immunohistochemical analysis of the skin from these mice showed a significant increase in the number of $p53^+$ basal epidermal cells in the skin of UVB-exposed animals after 1 week of UVB exposure compared with non-irradiated controls ($P<0.004$; Figure 3b). There was a further significant increase in the number of $p53^+$ cells in UVB-exposed CD4-depleted animals ($P<0.0002$) compared with UVB-exposed control IgG-injected animals. Individual $p53^+$ epidermal cells represented cells in which DNA damage resulting from exposure to UVB and to local inflammatory mediators has occurred. The p53 staining in these cells demonstrates 53 stabilization that occurs acutely in response to DNA damage.

Neutrophils contribute to increases in $p53^+$ induction in UVB-exposed skin

To determine the contribution of increased inflammation to the observed increases in p53 induction in the skin of CD4-depleted mice, Skh-1 mice ($n=5$ per group) were selectively depleted of neutrophils via injection with the neutrophil-depleting antibody LY6G before UVB exposure. Mice received three UVB exposures on non-consecutive days and skin was harvested 24 hours after the last exposure. Immunohistochemical analysis demonstrated a significant increase ($P<0.0006$) in the numbers of dermal infiltrating neutrophils in control animals at 1 week following UVB exposure (UV; Figure 4a). This increase was significantly inhibited ($P<0.0008$) in the skin of mice selectively depleted of neutrophils before exposure to UVB (UV/Ly6G). P53 immunohistochemical analysis of the skin of these mice demonstrated a significant increase in the number of $p53^+$ epidermal cells in UV-exposed skin (UV; Figure 4b; $P<0.0002$). This number was significantly decreased in the skin of mice selectively depleted of neutrophils before exposure to UVB ($P<0.003$; UV/anti-Ly6G).

CD4 depletion enhances skin tumor development in response to chronic UVB exposure

Skh-1 hairless mice ($n=40$) were exposed to 2240 J/m^2 UVB three times weekly for 10 weeks. At week 11, selective depletion of $CD4^+$ cells was initiated. This treatment strategy was designed to mimic the situation of midlife transplant patients who have had substantial UVB exposure before transplantation and therapeutic immunosuppression. Mice were randomized into one of four groups ($n=10$) and either (1) injected with control IgG antibodies and treated topically with (a)acetone ($n=10$; UV/IgG/Ace) or (b) celecoxib ($n=10$; UV/IgG/CX) or (2) injected with the CD4-depleting antibody and treated topically with (a) acetone ($n=10$; UV/anti-CD4/Ace) or (b) celecoxib ($n=10$; UV/anti-CD4/CX). Acetone or celecoxib was applied immediately following each UVB exposure. Mice continued to be exposed to UVB

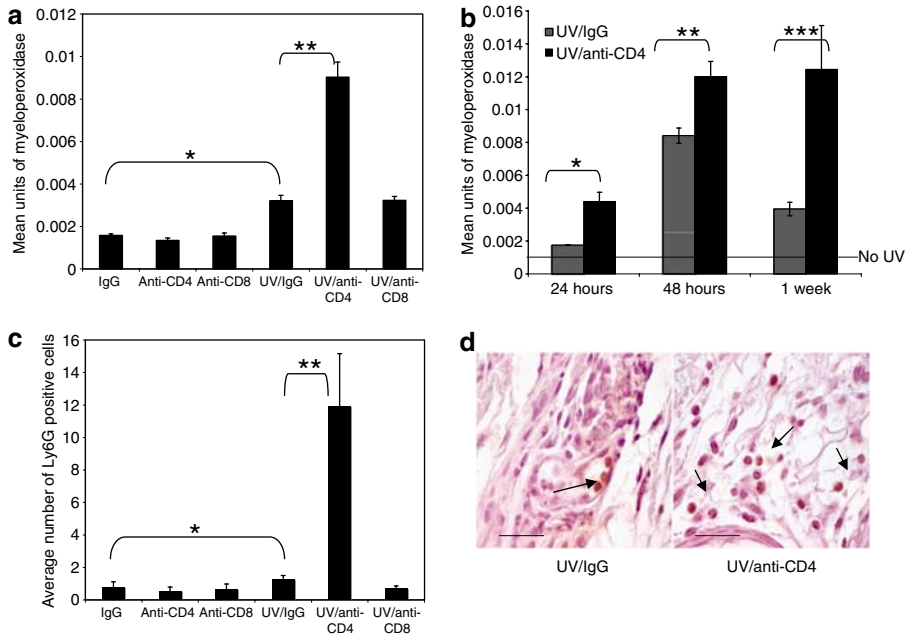

Figure 2. Selective CD4$^+$ cell depletion increases and prolongs UVB-induced MPO levels. The MPO assay was used to evaluate effects of CD4 and CD8 depletion on UVB-induced cutaneous inflammation (**a**) and to evaluate differences in the onset and length of the inflammatory response (**b**). Numbers of infiltrating LY6G positive neutrophils were counted in ten $\times 60$ fields per section (**c**). (**d**) Representative photomicrographs of LY6-G staining taken at $\times 100$ magnification (bar $= 20\,\mu$m). Error bars indicate SEM. (**a**) *$P<0.01$, **$P<0.004$; (**b**) *$P<0.001$, **$P<0.004$, ***$P<0.005$; (**c**) *$P<0.02$, **$P<0.04$.

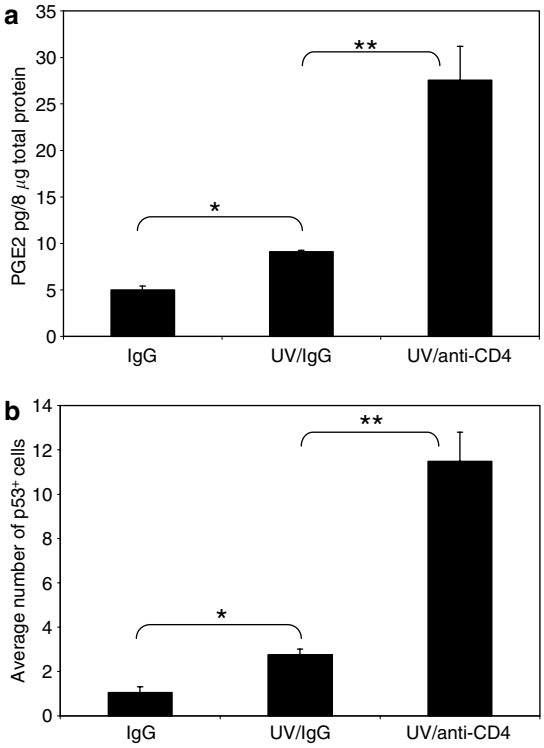

Figure 3. Effect of selective CD4$^+$ cell depletion on PGE_2 production and p53 induction in response to acute UVB exposure. PGE_2 levels (**a**) in skin isolated from mice injected with control IgG antibody or anti-CD4 antibody before acute UVB exposure were quantitated using an enzyme-linked immunosorbent assay. *$P<0.002$; **$P<0.006$. (**b**) Graphical representation of %p53$^+$ cells in the epidermis of treated mice. *$P<0.04$; **$P<0.0002$. Error bars indicate SEM.

and topically treated three times a week for the remainder of the study. Un-irradiated age-matched, antibody-injected controls ($n=5$) did not develop any tumors. Tumors greater than 1 mm in size per mouse were counted weekly beginning at week 14. At week 20, animals in the UVB/anti-CD4/Ace group began to exhibit a significant increase in tumor number compared with the UVB/IgG/Ace control group (Figure 5, $P<0.05$). This increase in tumor number remained evident until the time of killing at 25 weeks. On the basis of histologic examination, tumors included a spectrum of papillomas and SCC. Topical treatment with the anti-inflammatory drug celecoxib resulted in a significant ($P<0.05$) decrease in the number of tumors that developed regardless of immunosuppressive status in both IgG-injected control mice and CD4-depleted mice.

DISCUSSION

In humans, the CD4$^+$ T cell is the predominant lymphocyte infiltrating the skin in response to UV exposure (Di Nuzzo *et al.*, 1998). The presence of this cell type in the skin suggests that CD4$^+$ T cells play a critically important role in the acute cutaneous response to UVR. In general, it has been thought that infiltrating T cells contribute to the acute inflammatory response. However, to date no studies have specifically examined the potential link between CD4$^+$ cell function and acute UV-induced cutaneous inflammation, characterized by neutrophil infiltration, prostaglandin production via the COX-2 pathway and changes in p53 levels.

This study found that systemic depletion of CD4$^+$ T cells resulted in a significant increase in UVB-induced cutaneous inflammation. Changes in the skin included increased Gr-1$^+$

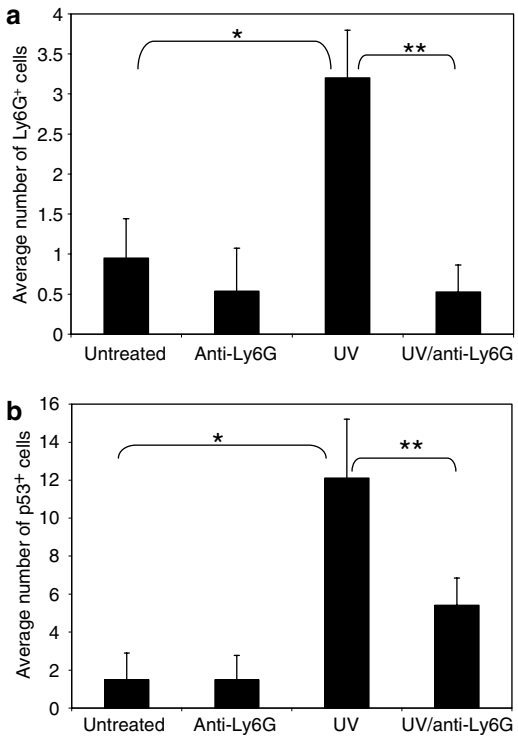

Figure 4. Selective depletion of neutrophils decreases UVB-induced epidermal p53. Skin of Skh-1 hairless mice selectively depleted of neutrophils was examined immunohistochemically and quantitated for (**a**) dermal neutrophil infiltration $*P<0.0006$, $**P<0.0008$; and (**b**) $p53^+$ epidermal cells. $*P<0.0002$; $**P<0.003$. Error bars indicate SEM.

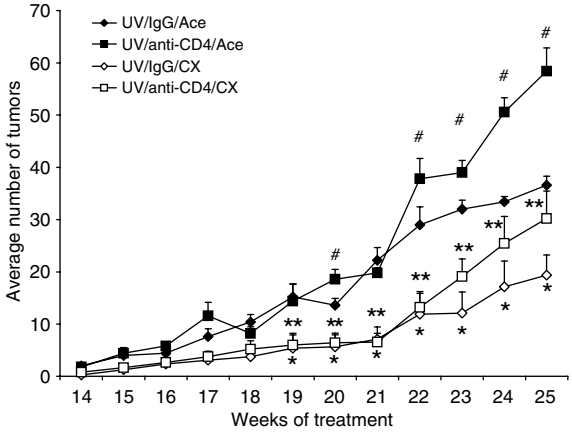

Figure 5. Effect of $CD4^+$ cell depletion on UVB-induced tumor development. Mice were exposed to 2240 J/m^2 UVB three times weekly for 25 weeks. Beginning at week 11, mice were either injected with anti-CD4 antibodies ($n=20$) or control IgG antibodies ($n=20$) every 3 weeks. Half of the mice in each group were treated topically with 0.2 ml acetone or 0.2 ml celecoxib in acetone immediately following each UV exposure. $*P<0.05$ compared with UV/IgG/Ace; $**P<0.05$ compared with UV/IgG/Ace $***P<0.05$ compared with UV/anti-CD4/Ace-treated groups. Error bars indicate SEM.

cell infiltration and MPO activity, longer persistence of MPO activity, increased PGE_2 levels, and increased numbers of $CD8^+$ cells. Gr-1^+ cells are typically considered neutrophils, although it is important to note that a small population of monocytes/macrophages involved in inflammation also express an intermediate level of Gr-1 (Henderson *et al.*, 2003). Hematoxylin and eosin analysis indicates that the infiltrating cells are predominantly polymorpho-mononuclear cells (neutrophils). Indeed, segmented nuclei are present in the Gr-1^+ cells (Figure 2d). However, this does not preclude a role for macrophages in this response. Further, although we cannot completely rule out the contribution of Gr-1-intermediate monocytes/macrophages, the depletion protocol used in this study depletes Gr-1-high but not Gr-1-intermediate cells (Miura *et al.*, 2003). We therefore believe that the Gr-1^+ cells are predominantly neutrophils.

Although the exact mechanism by which $CD4^+$ cell depletion enhances the inflammatory response is not known, we suggest the following sequence of events: UVB induces keratinocytes to produce chemotactic factors that recruit neutrophils and potentially macrophages. The infiltrating neutrophils produce reactive oxygen and nitrogen intermediates and inflammatory cytokines that attract T cells. $CD4^+$ T cells then infiltrate the skin where they act to dampen inflammation.

CD4 depletion also resulted in increased induction of p53 in basal epidermal cells following short-term UVR exposure. Systemically depleting neutrophils by injecting neutrophil-depleting antibody before UVB exposure significantly decreased the number of $p53^+$ basal epidermal cells. Thus, at least a portion of the p53 induction in our CD4-depleted groups may result from increased neutrophil infiltration. Increased induction of p53, evident immediately after UV exposure, is generally thought to reflect increased DNA damage leading to p53 stabilization in keratinocytes (reviewed in Melnikova and Ananthaswamy, 2005). A probable explanation of our observations would thus be that increased cutaneous inflammation in CD4-depleted animals led to increased production of DNA-damaging reactive oxygen species, resulting in increased DNA damage to keratinocytes. However, in the skin of animals exposed to UVR for 1 week, we were not able to detect immunohistochemically significant differences in 8-oxo-deoxyguanosine adducts, which result from increased reactive oxygen species production or cyclopyrimidine dimers among the treatment groups (data not shown).

Previous studies demonstrated a positive correlation between increased patient survival and the presence of lymphocytes in tumors in a variety of organs including colon, breast, and skin (Clemente *et al.*, 1996; Menard *et al.*, 1997; Naito *et al.*, 1998). Epidemiological studies in humans have correlated systemic immunosuppression and decreased lymphocyte function with increased skin cancer risk (Otley *et al.*, 2001). In this study we show that chronic UVB exposure combined with selective depletion of $CD4^+$ cells resulted in an increased tumor burden compared with control UVB-exposed IgG-injected animals. There were no differences in tumor size or grade between the treatment groups. Other murine studies have also suggested a role for $CD4^+$ T cells in modulating skin tumors. Using an orthotopic model Cavanagh and Halliday (1996) demonstrated that during the

development of murine UV-induced skin tumors, dendritic epidermal T-cell inhibition of $CD4^+$ T-cell activation played a role in the tumor escaping immune-mediated destruction. Moreover, $CD4^+$ T cells are important in inducing regression of both actinic keratotic lesions (SCC precursors) and orthotopic SCC (Halliday *et al.*, 1995; Patel *et al.*, 1995; Kondo *et al.*, 2002; Byrne and Halliday, 2003).

There is a strong link between inflammation and carcinogenesis. Normally, inflammation is an essential and self-limiting homeostatic mechanism. However, dysregulation of the inflammatory response can actually augment tumor formation. Epidemiological as well as basic research studies have demonstrated that the inflammatory response seen following both brief periods of high intensity sun exposure as well as chronic sun exposure plays a critical role in the formation of NMSC (Marks *et al.*, 1990; Gallagher *et al.*, 1995). Thus, it is possible that depletion of $CD4^+$ T cells and the subsequent increase in cutaneous inflammation that we observed in response to UVB may have modified the subsequent development of skin cancer. In our studies, topical treatment with the anti-inflammatory drug celecoxib significantly inhibited tumor development supporting the hypothesis that increased levels of inflammation in the skin contributed to the increased tumor number seen in our studies.

We have demonstrated that specific depletion of $CD4^+$ lymphocytes enhances susceptibility to UVB-induced SCC in a murine model of UVB-induced NMSC. Both increased UVB-induced cutaneous inflammation and decreased tumor immunosurveillance may contribute to enhanced skin tumor development in CD4-depleted mice. A question not addressed by this study is the potential contribution of different $CD4^+$ cell subpopulations to the effects we observed. As we depleted all $CD4^+$ cells, the exact cell responsible for the increase in UVB-mediated inflammation and tumor number has not yet been clarified. However, this study suggests an important and unexpected role for $CD4^+$ cells in maintaining cutaneous homeostasis and modulating inflammation. Interestingly, our data indicate that although normally $CD8^+$ cells do not infiltrate the epidermis in large numbers in response to UVB, their numbers are significantly increased in the absence of $CD4^+$ cells. This suggests that a balance between the two cells types may be necessary to regulate cutaneous inflammation. A clearer understanding of the effects of immunosuppression on the cutaneous inflammatory response and on skin cancer initiation, growth, and transformation into SCC would benefit not only immunocompromised patients but the general population as well.

MATERIALS AND METHODS

Animals

Outbred female Skh-1 hairless mice (6–8 weeks, 26–28 g, Charles River, Wilmington, MA) were housed in the vivarium at The Ohio State University according to the requirements established by the American Association for Accreditation of Laboratory Animal Care. The appropriate Institutional Animal Care Utilization Committee approved all procedures before beginning the study. Mice were housed on a 12-hour light/dark cycle and allowed RO water with Baytril 100 (Bayer, Shawnee Mission, KS) at a 40–50 mg/kg dose Baytril per mouse per day and standard lab chow *ad libitum*.

Acute UVB exposure and $CD4^+$/$CD8^+$ cell depletion

For the acute 1 week study, CD4-depleted ($n=10$), CD8-depleted ($n=10$), and IgG-control mice ($n=10$) were exposed on three non-consecutive days dorsally to one minimal erythemic dose (1 MED) of UVB (2240 J/m^2 as determined by a UVR meter (UVP Inc., Upland, CA) emitted by Phillips FS40UVB bulbs (American Ultraviolet Company, Lebanon, IN) that were fitted with Kodacell filters (Eastman Kodak, Rochester, NY) to ensure the removal of UVC wavelengths (290–320 nm). The GK1.5 monoclonal antibody (anti-CD4; Ligocyte Pharmaceuticals, Bozeman, MT) was used to deplete $CD4^+$ cells selectively. The YTS169 monoclonal antibody (Qin *et al.*, 1990) (anti-CD8, used with permission from Dr. H. Waldman, prepared by Ligocyte Pharmaceuticals) was used to selectively deplete $CD8^+$ cells. GK1.5 was injected i.p. in two doses of 0.5 mg/mouse 2 days apart, with the last injection being 24 hours before UVB irradiation, based on previous work using the GK1.5 antibody (Orosz *et al.*, 1997). YTS169 was injected i.p. in three doses of 0.1 mg/mouse on days −3, −2, and 0, with day 0 being the first day of UVB irradiation, based on published work using this antibody (Kish *et al.*, 2005). Control rat IgG (Sigma-Aldrich, St Louis, MO) was injected i.p. in two doses of 0.5 mg/mouse 2 days apart, with the last injection being 24 hours before UVB irradiation. The level of systemic $CD4^+$ and $CD8^+$ cell depletion per mouse was monitored by flow cytometry. The depletion protocols were sufficient to deplete $>95\%$ of the circulating $CD4^+$ or $CD8^+$ T cells for at least 2 weeks (data not shown). The antibodies and isotypic controls used to stain the cells for flow cytometry were CD3-FITC, CD4-PE, CD8-Cy-Chrome, FITC-rat IgG2bκ isotype control, PE-rat IgG2bκ isotype control and Cy-Chrome-rat IgG2aκ isotype control (Pharmingen, BD Biosciences, San Diego, CA). The animals were killed 24 hours after their last UVB exposure.

Chronic UVB exposure and $CD4^+$ cell depletion

For the chronic study, 40 animals were exposed to 1 MED of UVB on three non-consecutive days per week for 25 weeks, then divided into injection groups. At the beginning of week 11, animals were randomized and injected with either CD4-depleting antibody ($n=20$) or control IgG ($n=20$) as follows: Skh-1 hairless mice were injected with GK1.5 or control rat IgG i.p. in two doses of 0.5 mg/mouse 2 days apart every 3 weeks. To determine the systemic level of T lymphocyte depletion, white blood cells from each animal were isolated from peripheral blood collected by retro-orbital bleeding before the first T-cell-depleting antibody injection, every 6 weeks during the chronic study and at the time of killing. The antibodies and correct isotypic controls used to stain the cells for flow cytometry were CD3-FITC, CD4-PE, FITC-rat IgG2bκ isotype control, and PE-rat IgG2bκ isotype control (Pharmingen). Half of the mice in each injection group were treated topically immediately following UVB exposure with 0.2 ml of either the vehicle, high-pressure liquid chromatography grade acetone (Sigma-Aldrich), or 0.5 mg Celecoxib (Pfizer, New York, NY) dissolved in 0.2 ml acetone. To track changes in tumor number and size, tumors larger than 1 mm were measured weekly, using digital calipers, by an observer blinded to the experimental groups beginning at 14 weeks.

Acute UVB exposure and LY6-G depletion

Neutrophils were depleted via i.p. injection of the Gr-1 (anti-Ly6G) antibody, a kind gift of Dr R Fairchild (Cleveland Clinic Foundation). Anti-Ly6G antibody was diluted in phosphate-buffered saline (PBS) and injected i.p. 100 μg/mouse ($n=6$) on days -1, $+1$, $+3$, $+5$, with day 0 being the day the animals were first exposed to UVB. Control mice ($n=6$) received antibody injection with no UVB exposure. Mice were exposed for 1 week on three non-consecutive days to 2240 J/m^2 UVB and killed 24 hours following the last UVB exposure.

MPO Assay

MPO activity was assessed as described previously (Tober *et al.*, 2006). Briefly, a 10-mm^2 section of dorsal skin tissue was collected in 0.5% hexadecyltrimethylammonium bromide (Sigma) in 50 mM phosphate buffer. Tissues were homogenized and sequentially sonicated, frozen in liquid nitrogen, and thawed three times. The samples were centrifuged and the supernatants were collected. About 290 μl of o-dianisidine dihydrochloride at 0.167 mg/ml (Sigma) and 0.0005% hydrogen peroxide in phosphate buffer was added to 10 μl supernatant of each sample. MPO activity was measured over a 5 minutes period at 450 nm with a programmable microplate reader (Molecular Devices, Menlo Park, CA). The data are expressed as mean units of MPO activity per experimental group.

PGE_2 EIA

A 10-mm^2 section of dorsal skin tissue from each mouse was collected and snap-frozen. Tissues were homogenized in 1 ml of protein homogenation buffer (60 mM Tris-HCl, 5 mM ethylene glycol bis(â-aminoethylether)-*N,N,N′,N′*,-tetraacetic acid, 5 mM ethylene-diaminetetraacetic acid, 300 mM sucrose, 5 mM dithiothreitol, 200 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 10 mM sodium molybdate, 10 μM indomethacin), sonicated, and centrifuged. The supernatants were collected and protein was quantitated by Bradford assay (BioRad, Hercules, CA). A Caymen PGE_2 enzyme immunoassay (EIA) (Cayman Chemical, Ann Arbor, MI) was used to determine the concentrations of PGE_2. Eight micrograms of total protein was used for the EIA that was performed as outlined in the manufacturer's protocols.

Immunohistochemical analysis of $CD4^+$ and $CD8^+$ cells in the skin

Frozen sections of skin were used for immunohistochemical localization of $CD4^+$ or $CD8^+$ cells to verify whether the decrease in peripheral blood also occurred in the skin. Immediately following killing, skin sections (0.5 cm^2) were placed in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) and stored at -80°C. Tissue sections (10 μm) were cut and mounted onto Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA). The slides were air-dried for 30 minutes at room temperature, fixed in cold acetone for 10 minutes, and air-dried again. The sections were rinsed in Tris-buffered saline Tween-20 (TBST) for 5 minutes (TBST: 30.8 ml of 5 M NaCl, 100 ml Tris (pH 7.5), 1 ml Tween-20, final volume of 1 l). The sections were blocked for endogenous peroxidases using DAKO peroxidase block (DakoCytomation, Carpinteria, CA) for 5 minutes, rinsed with dH_2O and TBST. An avidin-biotin block (Vector Laboratories, Burlingame, CA) was used as directed and the sections were rinsed with TBST. DAKO Serum-free Protein Block (DakoCytomation) was added for 10 minutes. Purified anti-mouse CD4 (L3T4 Clone RM4-5 from Pharmingen) or purified anti-mouse CD8α (Ly-2 Clone 53-6.7 from Pharmingen) was added at 1:20,000 (CD4) or 1:15,000 (CD8) in DAKO Ab diluent (DakoCytomation) and incubated overnight at 4°C in a humid chamber. The sections were rinsed with TBST and biotinylated rabbit anti-rat IgG (Vector Laboratories) was applied at 1:200 (Vector Laboratories) for 30 minutes at room temperature. The sections were rinsed with TBST, ABC Elite was added (Vector Laboratories) for 1 hour at room temperature and the slides were rinsed with TBST, then Millipore water. Diaminobenzidine (DAB) (DakoCytomation) was added to each slide for 5 minutes. The sections were rinsed with Millipore water, methyl green (Vector Laboratories) was added for 5 minutes, and the sections were rinsed in 95% ethyl alcohol, dehydrated, and coverslipped. The positive epidermal cells (in or touching the epidermis and not located in the hair follicle) in ten $\times 60$ fields per section were determined using a Nikon microscope. The averages used in the graph were the averages of each experimental group ($n=10$), with the SE.

Immunohistochemical detection of Ly6-G

Immediately following sacrifice, skin sections (0.5 cm^2) were placed in 10% neutral-buffered formalin for 2 hours, washed with PBS, processed, and embedded in paraffin blocks. Tissue sections (5 μm) from paraffin blocks were cut and mounted onto Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA). The tissue sections were then deparaffinized using Clear-Rite 3 (Richard-Allan Scientific, Kalamazoo, MI) and rehydrated in a graded series of alcohols. Following rehydration, tissues were rinsed in automation buffer (Biomeda, Foster City, CA) and blocked with casein (Vector Laboratories) for 30 minutes. After every step that followed, slides were rinsed in automation buffer. The tissue was incubated with a 1:400 dilution of monoclonal rat anti-mouse Ly-6G (Pharmingen) or appropriate isotypic control antibody overnight at 4°C. The tissue was washed and incubated with a 1:400 dilution of biotinylated rabbit anti-rat secondary antibody (Vector Laboratories) for 30 minutes at room temperature, followed by an avidin/biotinylated horseradish peroxidase complex (ABC Elite, Vector Laboratories) for 30 minutes at room temperature. The tissue was incubated with DAB (Vector Laboratories) for 5 minutes, rinsed with distilled water and counterstained with hematoxylin 2 (Richard Allan Scientific, Kalamazoo, MI) for 0.5 minute. The sections were then dehydrated, mounted, viewed, and photographed. The numbers of Ly6G-positive dermal neutrophils in 10 $\times 60$ fields per section per mouse ($n=10$) were counted. The averages per experimental group were graphed with SE.

Immunohistochemical detection of p53 protein

Immediately following sacrifice, skin sections (0.5 cm^2) were placed in 10% neutral-buffered formalin for 2 hours, washed with PBS, processed, and embedded in paraffin blocks. Tissue sections (5 μm) were cut and mounted onto Superfrost Plus microscope slides (Fisher Scientific). The tissue sections were then deparaffinized using Clear-Rite 3 (Richard-Allan Scientific) and rehydrated in a graded series of alcohols. Following rehydration, an antigen retrieval process was performed by placing the slides in 95°C antigen-unmasking fluid for 20 minutes (Vector Laboratories) followed with a 20-minute cooling period. The sections were washed in automation buffer (Biomeda

Corp., Burlingame, CA) and blocked with casein for 30 minutes (Vector Laboratories). The tissue was incubated with primary polyclonal rabbit anti-mouse p53 antibody (Vector Laboratories) diluted 1:300 in casein or rabbit IgG for 90 minutes at room temperature. The tissue was washed with automation buffer, then incubated for 30 minutes at room temperature with rabbit link solution for 30 minutes (BioGenex, San Ramon, CA) and then label solution for 30 minutes (BioGenex). After incubation with DAB for 6 minutes and a final wash in distilled water, the tissue was counterstained with hematoxylin, dehydrated and mounted. The number of $p53^+$ basal epidermal cells in ten $\times 60$ fields per section was determined using a Nikon Eclipse E400 inverted microscope with a DXM1200 digital camera.

Statistical Analysis

A biostatistician was consulted for appropriate numbers of animals per experimental group. Student's *t*-test was used to study the main effect of each treatment factor on each response measured, where $P \leqslant 0.05$ is significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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